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Decrease in the cattle tick *Rhipicephalus microplus* biological parameters using anti-subolesin peptide antibodies by artificial capillary feeding

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Abstract. Ticks represent a threat to animal health worldwide and are considered the second most important vectors of human diseases. The main method of control of ticks has been the usage of chemical products; however, the use of acaricides has resulted in some serious drawbacks such as acaricide-resistant ticks and environmental pollution. As a result the use of immunological control using tick proteins is suggested as an alternative to control tick populations. The protocols used to test the ticks to antigens, needs a complementary method that would allow control to be carried out in external conditions. In this context, the use of the *in vitro* capillary feeding represents a low cost alternative to test candidate antigens allowing to get important data on the protective effect during interactions between antigen-antibody. In order to evaluate the effect in biological parameters of female *R. microplus* ticks by artificial feeding with bovine blood containing anti-subolesin peptide IgG's obtained at different times after immunization. Results of this study showed the effect on weight of ticks and oviposition due to antibodies obtained at weeks 3, 5 and 7 after immunization. The results proved that anti-subolesin peptide IgG´s at week 7 demonstrated better effect reducing tick weight by 45% and oviposition by 71% ($P<0.05$) with respect to tick fed on blood with preimmune serum. These results strongly suggested that the negative effects in cattle tick biological parameters were the result of the protective antibodies against subolesin peptide. Finally, the artificial feeding of ticks should be used to evaluate antigen-specific antibodies avoiding external factors.

INTRODUCTION

The cattle tick *Rhipicephalus microplus* is the most important ectoparasites affecting the cattle industry in tropical and subtropical areas around the world (Almazan *et al*., 2010). Infestations with *R. microplus*, economically impact cattle production by reducing weight gain, milk production and also by transmitting pathogens that causes babesiosis and anaplasmosis (Merino *et al*., 2011; Merino *et al*., 2013). It is estimated that the worldwide economic impact of *R. microplus* is approximately 2.5 billion US dollars per year (Lew-Tabor *et al*., 2014).

Currently, acaricides are the principal methods of tick control strategies. However, due to the rapid appearances of resistants in tick populations and the presence of chemical residues in meat and milk are of health concerns that emphasize the need for novel control methods, such as tick vaccine (Willadsen & Mckenna, 1991; Willadsen *et al*., 1996; Imamura *et al*., 2005).

Vaccination programs with the commercial vaccines containing the recombinant *R. microplus* Bm86 gut antigen, Gavac® and Tick-GARD® have been established in several regions of Australia and Latin America, however, different levels of

efficacy to *R. microplus* strains have been experienced and sequence variations in the target protein among different strains that have been found to be associated with variable efficacy (García-García *et al.*, 1999; de la Fuente *et al.*, 1999; Canales *et al.*, 2009a).

Other tick protective antigens, such as subolesin, was discovered in *Ixodes scapularis* as a structural and functional ortholog of insects and vertebrates akirins and functions as a transcription factor in regulation of gene expression (Almazán *et al.*, 2003; de la Fuente *et al.*, 2005, Galindo *et al.*, 2009). Subolesin has been extensively studied by different research groups in previous vaccination trials, demonstrating protective efficacy against tick infestations, reduced fertility and/or survival of several arthropod vector species and vectorial capacity of ticks (Harrington *et al.*, 2009; Canales *et al.*, 2009b; Almazán *et al.*, 2010; Prudencio *et al.*, 2010; de la Fuente *et al.*, 2013; Merino *et al.*, 2013; Shakya *et al.*, 2014). Recently, Lagunes *et al.* (2016), synthesized a recombinant peptide based on linear B-cell and conformed discontinuous epitopes that predicted into the sequence of the Subolesin protein by bioinformatics analysis, which was evaluated in a preliminary cattle immunization test against *R. microplus*. This evaluation demonstrated reduction on tick numbers and egg hatching, but had no effect on *R. microplus* tick weight and oviposition. The aim of the study was to evaluate the protective effect using one of the lowest cost techniques for testing vaccine by *in vitro* capillary feeding (Gonsioroski *et al.*, 2012; Antunes *et al.*, 2014; Abel *et al.*, 2016) with purified bovine polyclonal antibodies IgG's against the subolesin peptide produced in cattle in order to provide results more closely resembling vaccine protective potential.

MATERIALS AND METHODS

Tick strain

The present study was carried out in the Laboratory of Parasitology and Molecular Biology of the Faculty of Veterinary Medicine and Animal Science, from the University of Tamaulipas. The biological material used was the *R. microplus* "Media Joya" tick strain, belonging to the CENID-PAVET, INIFAP, and was kept under controlled conditions since 2009 at the FMVZ-UAT. Originally, this tick strain was collected from infested cattle in Tapalpa, Jalisco, Mexico.

Production and purification of IgG's

In the present study, antibodies against subolesin peptide were obtained from a previous study (Lagunes *et al.*, 2016). For the production of antibodies, two bovines were immunized with 2 doses (days 0, 30) containing 100 µg/dose of purified recombinant subolesin protein in 1 ml Montanide ISA 50 V adjuvant (Seppic, Paris, France). Cattle were injected subcutaneously with 2 ml/dose using a 5 mL syringe and an 18 G needle. Blood was collected before the injection (Preimmune) as well at weeks 3, 5 and 7 after the first and second immunization to prepare preimmune and immune serum, respectively. Serum aliquots were kept at 4°C for immediate use or at –20°C for long-term storage. IgG's were purified from serum samples using the Montage Antibody purification kit and spin columns with PROSEP-A Media (Millipore, MA, USA) following the manufacturer's recommendations.

Artificial feeding

Artificial feeding of ticks was carried out with partially engorged female *R. microplus* ticks which were recovered manually from calves 20–21 days after infestation with larvae. Afterwards, they were cleaned, weighed and fixed on expandable polystyrene plates (19 x 10 cm) with double-sided adhesive tape (3 M, St. Paul, MN, USA). Females were discarded if they had damaged mouthparts or if their weight did not lie between 20 and 60 mg. Sodium citrated treated bovine blood from uninfected animals were used to fill microhematocrit capillary tubes (75 x Ø1.5 mm) that were placed over the ticks' mouthparts. Tubes were replaced every 2–3 h, as described previously (Gonsioroski *et al.*, 2012; Antunes *et al.*, 2014). Female ticks were divided in experimental groups,

each formed by 10 individuals and fed 28 h with blood supplemented with 1 mg/ml of preimmune or antigen-specific purified IgG's. After the feeding, ticks were detached from the double-sided tape and were weighed again to determine tick weight increase during feeding. All ticks were then placed in Petri dishes and incubated at 27°C and 85% humidity for oviposition. Animal experiments were carried out in strict accordance with the Guide for Care and Use of Laboratory Animals for the University of Tamaulipas and the protocol was approved by the Committee on the Ethics of Animal Experiments (Permit no: CBBA-15-06).

Data collection and analysis

The biological parameters that were analyzed were tick gain weight during feeding (mg per tick) and egg production (weight of eggs per tick in mg). They were then compared between ticks fed with blood supplemented with antibodies against subolesin peptide and control ticks fed with bovine blood supplemented with preimmune serum by Student's t test with unequal variance ($P<0.05$). A correlation analysis was conducted in Microsoft Excel (version 12.0) to compare the weight gain and egg production of female ticks after artificial feeding with the anti-subolesin peptide antibody level at different times (preimmune and weeks 3, 5, 7).

RESULTS

Effect of subolesin-antibodies on tick weight

Capillary feeding experiments were conducted to evaluate the effect of antibodies against ticks that were fed with blood supplemented that contaided anti-subolesin peptide IgG's and it demonstrated higher reduction in tick weight after 3 weeks (62% reduction; 0.076 mg/tick on average). This effect could be due to the immunological response against the subolesin peptide and the effect is smaller but significant in the following weeks. At week 5 and 7 there was 40% and 45% reduction, respectively ($P<0.05$), when compared to ticks fed on blood with preimmune serum (Figure 1).

The results showed a difference in weight gain between groups fed on blood supplemented with preimmune and immune IGg's. In order to address this aim, a correlation analysis was carried out using the weight gain compared to antibody level obtained previously by Lagunes *et al.* (2016). This suggests that the reduction in the weight of female *R. microplus* ticks could be related to the presence of anti-subolesin peptide IgG's in the blood meal ingested. Interestingly, higher antibodies level ($OD_{405nm}=2.93$) does not correspond with the lowest tick weight (76 mg/tick) on average. However, these results demonstrated a

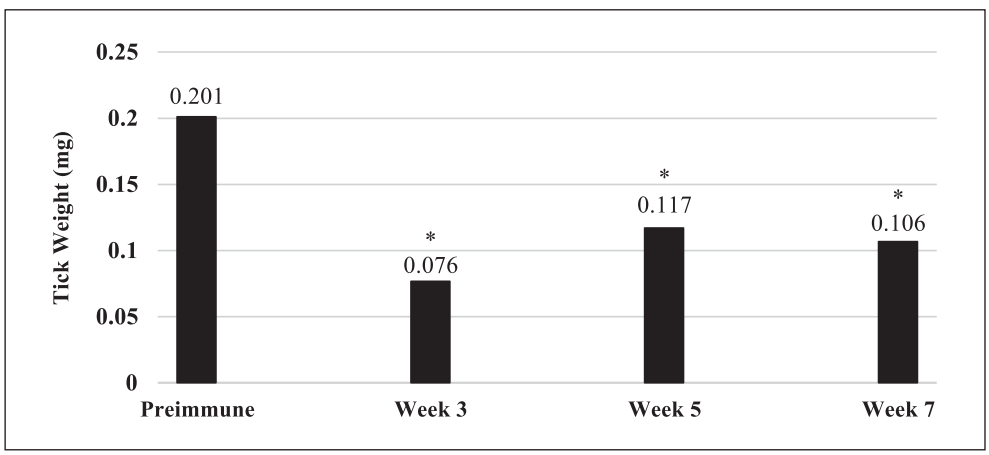

Figure 1. Effect of subolesin antibodies on tick weight. Ticks (N=10) were weighted before and after artificial feeding. The tick weight (mg/tick) was determined per group (week 3, 5, 7) and compared with preimmune serum by Student's t test (*$P<0.05$).

positive correlation between reduction in tick weight and antibody level in blood supplemented with anti-subolesin peptide IgG´s when compared to ticks fed on blood supplemented with preimmune serum (Figure 2).

Effect of antibodies against tick protein on oviposition

Egg production was evaluated two weeks after artificial feeding. A total of 21 ticks died without laying eggs. Oviposition was reduced in the groups fed on blood with anti-subolesin peptide; the most affected group for egg production (71% reduction) ($P<0.05$) was on the 7th week when compared to ticks fed on blood supplemented with preimmune serum; this effect could be due to the higher anti-subolesin peptide antibody level present in serum after immunization (Figure 3).

A direct correlation between antibody level and egg production was observed. Anti-subolesin peptide IgG's at week 7 (OD_{405nm}=2.93) were correlated with the lowest egg production (0.005 mg eggs/tick) after capillary feeding and indicated a

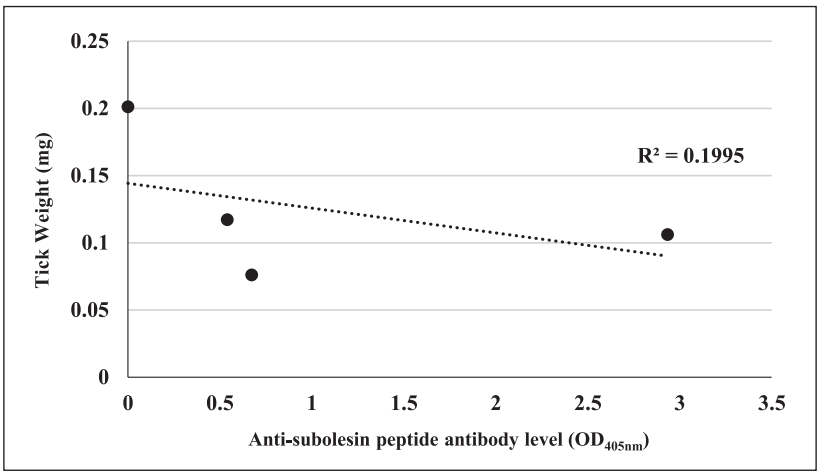

Figure 2. Anti-subolesin peptide antibody positively correlated with the reduction of tick weight. A correlation analysis was conducted using Microsoft Excel (version 12.0) between the tick female weight and anti-subolesin peptide antibody level at different time. The linear correlation coefficient (R^2) is shown.

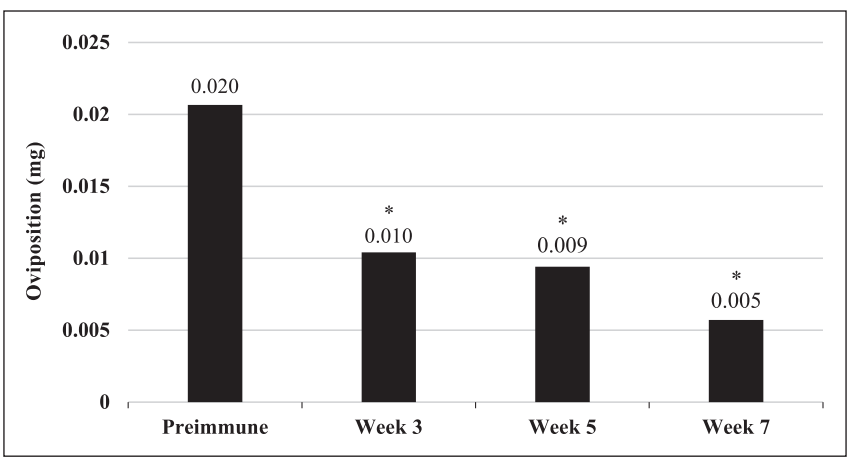

Figure 3. Effect of antibodies on tick oviposition. Ticks (N=10) were incubated for oviposition after feeding, the egg production (mg eggs/tick) was determined for each group (week 3, 5, 7) and compared with preimmune serum by Student's t test (*$P<0.05$).

positive correlation at weeks 3 and 5 showing significant differences when compared to group fed on blood mixed with preimmune serum (Figure 4). These results strongly suggest that the reduction in oviposition and was the result of the effect of the antibodies against subolesin peptide produced in cattle.

The effect of the anti-subolesin antibodies determined the reduction in the *in vitro* feeding capacity of ticks and the amount of eggs produced, while artificial feeding with pre-immune serum did not have a significant effect on any of the tick parameters analyzed (Figure 5). These results showed that while

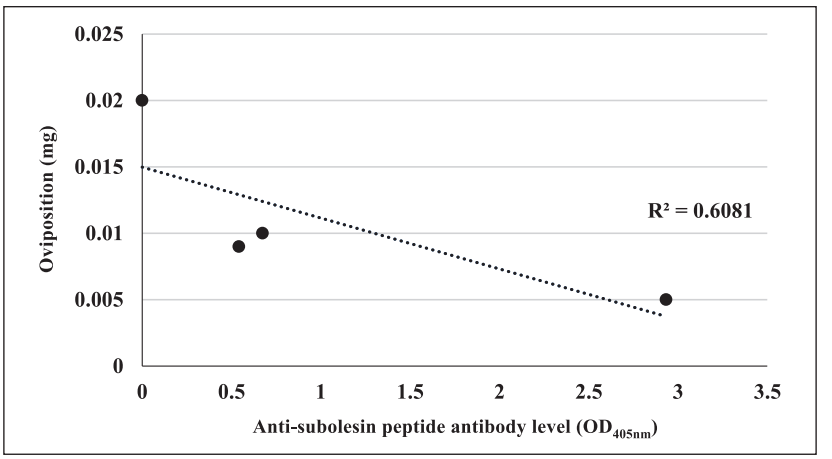

Figure 4. Anti-subolesin peptide antibody positively correlated with the reduction of oviposition. A correlation analysis was conducted using Microsoft Excel (version 12.0) between the oviposition and anti-subolesin peptide antibody level at different time. The linear correlation coefficient (R^2) is shown.

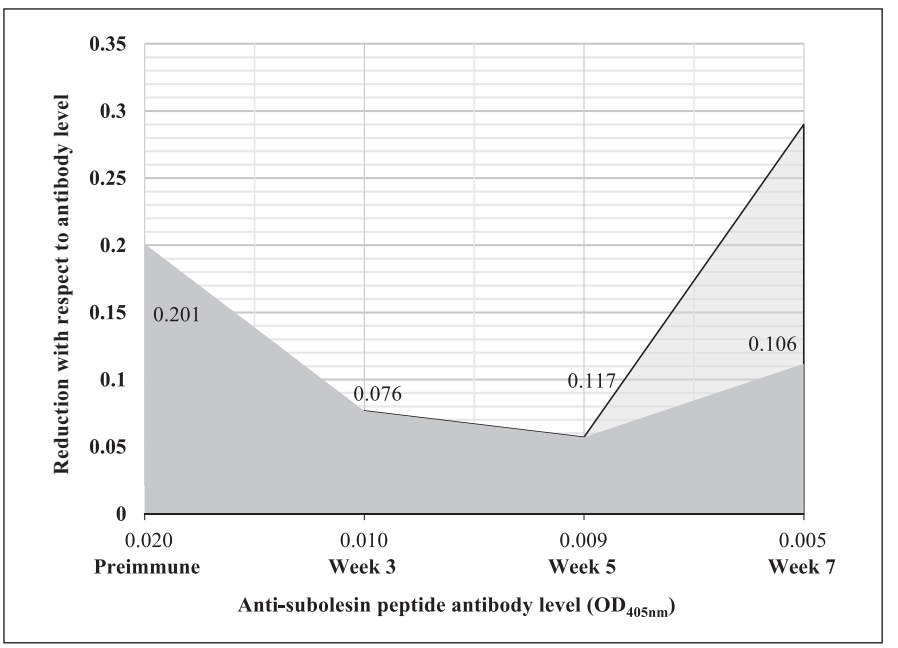

Figure 5. Antibody efficacy on the control of tick biological parameters. Comparison between the tick weight and oviposition of female ticks after feeding from both anti-subolesin peptide IgG´s and preimmune serum at different weeks with respect to antibody level.

the antibody level increase, tick weight and oviposition should decrease.

DISCUSSION

The use of artificial capillary feeding of ticks is a successful technique for the evaluation of antibodies directed against tick proteins that is involved in development, vital functions, and tick-pathogen interactions (Gonsioroski *et al.*, 2012; Antunes *et al.*, 2014; Abel *et al.*, 2016). This evaluation simulates the natural feeding process and allows the possibility to identify the tick protective antigens more closely. Tick vaccine could be a better strategy for control, than chemical products, as they are safer and free of residues to the host and for the environment (Patarroyo *et al.*, 2009; Aguirre *et al.*, 2016). Tick Subolesin has been studied extensively by several groups (Almazán *et al.*, 2010; de la Fuente *et al.*, 2011; Merino *et al.*, 2011, 2013; Shakya *et al.*, 2014). Vaccination with Subolesin has shown to have an effect on the control of *R. microplus* infestation, resulting in a reduction of female reproductive performance parameters (Almazán *et al.*, 2010; Merino *et al.*, 2013; Shakya *et al.*, 2014). However, the use of the Subolesin vaccine has not been totally successful since only 40–60% efficacy has been obtained in controlled infestations experiments (Almazán *et al.*, 2010; Merino *et al.*, 2013; Shakya *et al.*, 2014). A recent study using a reverse vaccinology approach to detect potentially immunogenic regions from the Subolesin protein of *R. microplus*, allows design and synthesized a peptide with antigenic properties to act faster (Lagunes *et al.*, 2016). However this peptide did not reduce tick weight and oviposition in ticks fed on subolesin peptide-vaccinated cattle in contrast to the resultsof previous vaccination trials in cattle (Merino *et al.*, 2013; Shakya *et al.*, 2014). However, the percent reduction is lower than other proteins evaluated in vaccination trials in cattle (de la Fuente *et al.*, 1999; Canales *et al.*, 2009a,c; Jeyabal *et al.*, 2010). Our results indicated a higher effect against tick weight and oviposition by using antigen-specific purified IgG's through artificial feeding. Our results showed a better effect than those previously obtained by vaccination and/or RNA interference (RNAi) in *R. microplus*, *Ixodes scapularis*, *Dermacentor variabilis* and *Amblyomma cajennense* (Patarroyo *et al.*, 2002; de la Fuente *et al.*, 2006; Abel *et al.*, 2008; Merino *et al.*, 2011; Parizi *et al.*, 2012) indicating that artificial feeding can provide ticks with the amount of blood mixed with antibodies necessary to affect biological parameters as tick weight and oviposition. As expected, anti-subolesin peptide antibody level at week 3 and 5 was similar, but in week 7, antibodies increased due to a specific anti-subolesin peptide IgG's that were predominant in serum. Elicited IgG antibodies positively correlated with the reduction of tick weight and oviposition of *R. microplus* after artificial feeding. These results strongly suggest as in previous experiments with Bm86, Bm95, Subolesin (Cobon *et al.*, 1995; de la Fuente *et al.*, 1998; Carreon *et al.*, 2012; Moreno-Cid *et al.*, 2013) that the reduction in cattle tick biological parameters was the result of the protective antibodies against Bm86, Bm95 and Subolesin in vaccinated cattles. However, the anti-tick effects evaluated in this study, were higher compared to results reported in previous experiments using artificial feeding with anti-subolesin IgG's possibly due to the immunogenic region that are being selected for the peptide design.

These results suggest that strong interactions between antigen-specific antibodies and specific domains present in the molecule (extracellular portion) could reduce the weight gain and egg production in female ticks by some unknown mechanisms. In theory, the amount of blood mixed with antibodies ingested by female ticks must be higher in order to produce damage by multiple cellular processes such as digestion, development and reproduction decreasing biological parameters. In conclusion, the current results present a higher percent of efficacy by using this experimental approach for the selection and design of candidate tick protective antigens. Other

immune mechanisms could be involved in vaccine protection and future studies can be used to characterize the immune response against such molecule or peptide.

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